Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray

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Although most eukaryotic mRNAs need a functional cap binding complex eIF4F for efficient 5′ end- dependent scanning to initiate translation, picornaviral, hepatitis C viral, and a few cellular RNAs have been shown to be translated by internal ribosome entry, a mechanism that can operate in the presence of low levels of functional eIF4F. To identify cellular mRNAs that can be translated when eIF4F is depleted or in low abundance and that, therefore, may contain internal ribosome entry sites, mRNAs that remained associated with polysomes were isolated from human cells after infection with poliovirus and were identified by using a cDNA microarray. Approximately 200 of the 7000 mRNAs analyzed remained associated with polysomes under these conditions. Among the gene products encoded by these polysome-associated mRNAs were immediate-early transcription factors, kinases, and phosphatases of the mitogen-activated protein kinase pathways and several protooncogenes, including c-myc and Pim-1. In addition, the mRNA encoding Cyr61, a secreted factor that can promote angiogenesis and tumor growth, was selectively mobilized into polysomes when eIF4F concentrations were reduced, although its overall abundance changed only slightly. Subsequent tests confirmed the presence of internal ribosome entry sites in the 5′ noncoding regions of both Cyr61 and Pim-1 mRNAs. Overall, this study suggests that diverse mRNAs whose gene products have been implicated in a variety of stress responses, including inflammation, angiogenesis, and the response to serum, can use translational initiation mechanisms that require little or no intact cap binding protein complex eIF4F.

Infection of cells by a variety of RNA viruses results in the selective inhibition of translation of host but not of viral mRNAs. For example, infection of cells with poliovirus, a cytoplasmic RNA virus, results in the modification of several translation initiation factors that are implicated in the efficient recruitment of 40S subunits to capped mRNAs. Specifically, the proteolysis of both forms of eIF4G, eIF4GI and eIF4GII (1), by virally encoded proteases results in inhibition of translation of most capped cellular mRNAs. In contrast, the translation of polioviral mRNA, which contains a 450-nt sequence in the viral 5′ noncoding region (5′NCR) that can recruit 40S subunits in the absence of intact eIF4F, is not inhibited. This sequence element was termed an ''internal ribosome entry site'' or ''IRES'' (2).

To date, a handful of cellular mRNAs containing IRES elements have been identified (reviewed in refs. 3 and 4). These cellular mRNAs can presumably recruit 40S ribosomes both via either their capped 5′ ends or their IRES elements. In addition, a few viral and cellular mRNAs that do not contain IRES elements have been shown to have a low requirement for intact eIF4F. Notably, late adenoviral (reviewed in ref. 5) and heat shock mRNAs (reviewed in ref. 6) have been shown to be translated when eIF4F is limiting, presumably because of the low amount of RNA structure in their 5′NCRs.

To identify natural mRNAs that can be translated at reduced eIF4F concentrations, mRNAs that were associated with polysomes in poliovirus-infected cells were identified by using a cDNA microarray. The results showed that at least 3% of the mRNAs were associated with fast-sedimenting polysomes in poliovirus-infected cells at a time when most of the two forms of eIF4G had been proteolyzed. Thus, translation initiation mechanisms that require little or no intact eIF4F, such as internal initiation, may be used for the selective and regulated translation of certain mRNAs.

Materials and Methods

Cells and Viruses. HeLa cells were maintained in DMEM supplemented with 10% calf serum and 1× antibiotic/antimycotic (GIBCO/BRL). HeLa cells were infected with poliovirus mutant 3NC202 with a multiplicity of infection of 30 plaque forming units/cell and were incubated at 37°C (4).

DNA Reagents. By using the RadPrime kit (GIBCO/BRL), radiolabeled DNA probes were generated from human actin (nucleotides 685–1,171) and c-myc (nucleotides 1,683–2,121) DNAs, and from the following Imageclones (Research Genetics, Birmingham, AL): Pim-1 (clone ID 428404); Cyr61 (clone ID 486700); B94 (clone ID 487045); CL100 (clone ID 417357); MAPKK3b (clone ID 471649).

RNA Isolation and Northern Blot Analysis. Total and polysomal RNA was isolated by using Trizol reagent (GIBCO/BRL) and was analyzed as described (4).

Preparation of Fluorescently Labeled cDNA for Microarray Analysis and Microarray Hybridization. RNA from polysomes (Fig. 5, fractions 8–10) was pooled from gradients derived from mock or poliovirus-infected cells and was used to generate fluorescently labeled DNA probes as described (7). In brief, 10 μg of the pooled RNA was converted into single-stranded cDNA by reverse transcription using an oligo-dT primer. Labeled probe was generated by using random nonameric DNA and Klenow DNA polymerase and was hybridized to a ''10K'' human cDNA microarray, containing ''named'' human genes, genes that seemed to be related to genes in other organisms and ''unnamed'' genes included on the human transcript map (www.ncbi.nlm.nih.gov/SCIENCE96/) (8). An image of the array was obtained by using a general laser confocal slide scanner and was analyzed by using SCANALYZE software (http://genome-www.stanford.edu/software). For each array, a single

Abbreviations: 5′NCR, 5′ noncoding region; IRES, internal ribosome entry site; MAP, mitogen-activated protein; MKP, MAP kinase phosphatase.

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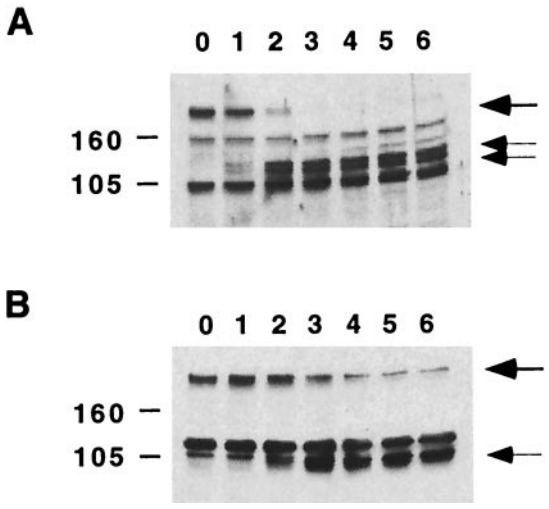

Fig. 1. Cleavage of eIF4GI and eIF4GII during poliovirus 3NC202 infection. Cell lysates (20 μg) collected at different times (hours; indicated at the top of the figure) after infection were separated by SDS/PAGE and were immunoblotted with antibodies directed against eIF4GI (*A*) or eIF4GII (*B*) (1, 27). Thick arrows indicate the migration of full-length eIF4G proteins, and thin arrows indicate the migration of the cleaved eIF4G products. Migration of known molecular weight markers is shown on the left.

multiplicative normalization factor was computed and applied to all Cy3/Cy5 ratios so that the median normalized Cy3/Cy5 ratio was 1.0. Images and processed data are available at http://genome-www.stanford.edu/polio.

Construction of Dicistronic Vectors. The dual luciferase construct (pRF) was created as follows: First, a *Bgl*II/*Xba*I fragment, containing the *Renilla* luciferase gene linked to the SV40 promoter, was excised from pRL-SV40 (Promega). The protruding ends were repaired by treatment with Klenow fragment, and the fragment was inserted into the *Sma*I site of pGL3 (Promega). Finally, an inactive encephalomyocarditis virus (EMCV) IRES, ≈440 nucleotides in length, (9) was amplified by PCR to yield a fragment with *Xho*I and *Eco*RI end sequences and then was inserted into the intercistronic region of plasmid pRF, at an *Eco*RI site seven base pairs upstream of the firefly luciferase gene, to yield plasmid pRΔDEF.

The 5′NCRs of Pim-1 (nucleotides 1–354) (10) and Cyr61 (nucleotides 105–224) (11) were amplified in PCR reactions using gene-specific 5′ primers containing *Eco*RI sites at their 5′-ends, and 3′ gene- specific primers that converted the AUG start codons to *Nco*I sites. Note that we have been unable so far to obtain a cDNA that contains a full-length 5′NCR of Cyr61. Plasmids containing Pim-1 or Cyr61 cDNA were used as templates in the PCR reactions. The amplified PCR products were digested with *Eco*RI and *Nco*I and were inserted into the dicistronic vector pRΔDEF.

Results

Intracellular Fate of Host Cell mRNAs in Poliovirus-Infected Cells. The inhibition of host cell mRNA translation has been found to coincide with the cleavage of the two forms of eIF4G, eIF4GI and eIF4GII, in virally infected cells (1). An 8-nt insertion in the 3′ noncoding region of poliovirus gave rise to mutant 3NC202, which has a defect in viral RNA replication that makes the inhibition of cap-dependent translation in infected cells more gradual than the inhibition observed in wild-type-infected cells (12, 13). Fig. 1*A* shows that all eIF4GI was proteolyzed after 3 hours of 3NC202 infection. Although the proteolysis of eIF4GII (Fig. 1*B*) remained incomplete throughout the infection, at least 50% of intact eIF4GII was degraded at 3 hours after infection. These data suggest that most of the cap binding protein complex eIF4F, containing either eIF4GI or eIF4GII, was nonfunctional at this time after infection. This notion was also supported by the findings that the amplitude and size of polysomes were greatly reduced and translation of most cellular mRNAs was inhibited at this time after infection (Fig. 5) (4).

The steady state levels of individual mRNAs from uninfected and infected cells were compared after hybridization of first strand cDNAs to a human 10K cDNA microarray (8). Remarkably, of the ≈7,000 genes for which reliable measurements were obtained, only 0.3% showed changes of 1.7-fold or more in their concentration in the cell at 3 hours after infection. Specifically, the intracellular levels of 18 mRNA species increased while the levels of 12 mRNAs decreased in infected cells.

Association of Cellular mRNAs with the Translation Apparatus in Poliovirus-Infected Cells. In contrast to the modest change in steady state mRNA concentration, the cellular mRNAs associated with polysomes changed dramatically. Our analysis revealed 230 mRNA species (i.e., 3% that were measured) were >2-fold enriched in the polysome-associated fractions of poliovirus-infected cells compared with mock-infected cells. Fig. 2 shows the polysomal association of mRNAs whose abundance did not change during poliovirus infection.

Only previously named genes are included in this analysis; mRNAs represented only by expressed sequence tags were omitted. Fig. 2*A* displays data from three mRNAs representative of most host cell mRNAs which were associated with polysomes from uninfected but not with polysomes from infected cells. Fig. 2 *B–I* lists the genes whose mRNAs were associated with polysomes from both uninfected and infected cells. Notably, this list includes mRNAs that encode proteins involved in nuclear-cytoplasmic transport of macromolecules (NUP153, Ran-binding protein 2), kinases and phosphatases involved in stress-activated p38 mitogen-activated protein (MAP) kinase pathways (MAPKK3b, MAP kinase phosphatase 1), protooncogenes (B94, Cyr61), and DNA binding proteins and receptors (TGFIII receptor, glucocorticoid receptor).

Of the twelve mRNAs whose intracellular levels increased in infected cells, nine correspond to named genes. All nine were found to be greatly enriched in the polysomal fractions from infected cells (Fig. 3). Several of these mRNAs encode so-called "immediate early transcription factors" ATF3, TIEG, c-myc, and MINOR (Fig. 3), whose expression is transcriptionally induced by serum. The experimental protocol in this study was designed to minimize effects of serum, and not all serum-responsive mRNAs are present in the list; therefore, the enhanced steady state levels of these mRNAs may reflect an antiviral response of the infected cell.

An independent assay was used to verify the relative levels and integrities of certain mRNAs in poliovirus-infected cells. Northern analysis showed that the amounts of actin, B94, MAPKK3b, and MAP kinase phosphatase 1 (Cl100) RNAs obtained from cells at 2 or 3 hours after infection were comparable to those from mock-infected cells (Fig. 4). In contrast, the amount of Cyr61 mRNA slightly increased and the levels of c-myc and Pim-1 mRNAs greatly increased during infection. These results all concur with the microarray data.

To analyze directly the polysomal association of some of the RNAs listed in Fig. 4, polysome gradients were prepared from mock-infected and virus-infected cells (Fig. 5). Gradient fractions 8–10 represent fractions in which five or more ribosomes were associated with each mRNA. Actin mRNA, efficiently translated in uninfected cells, redistributed from fraction 9 in gradients prepared from mock-treated cells to fractions 5–6 and

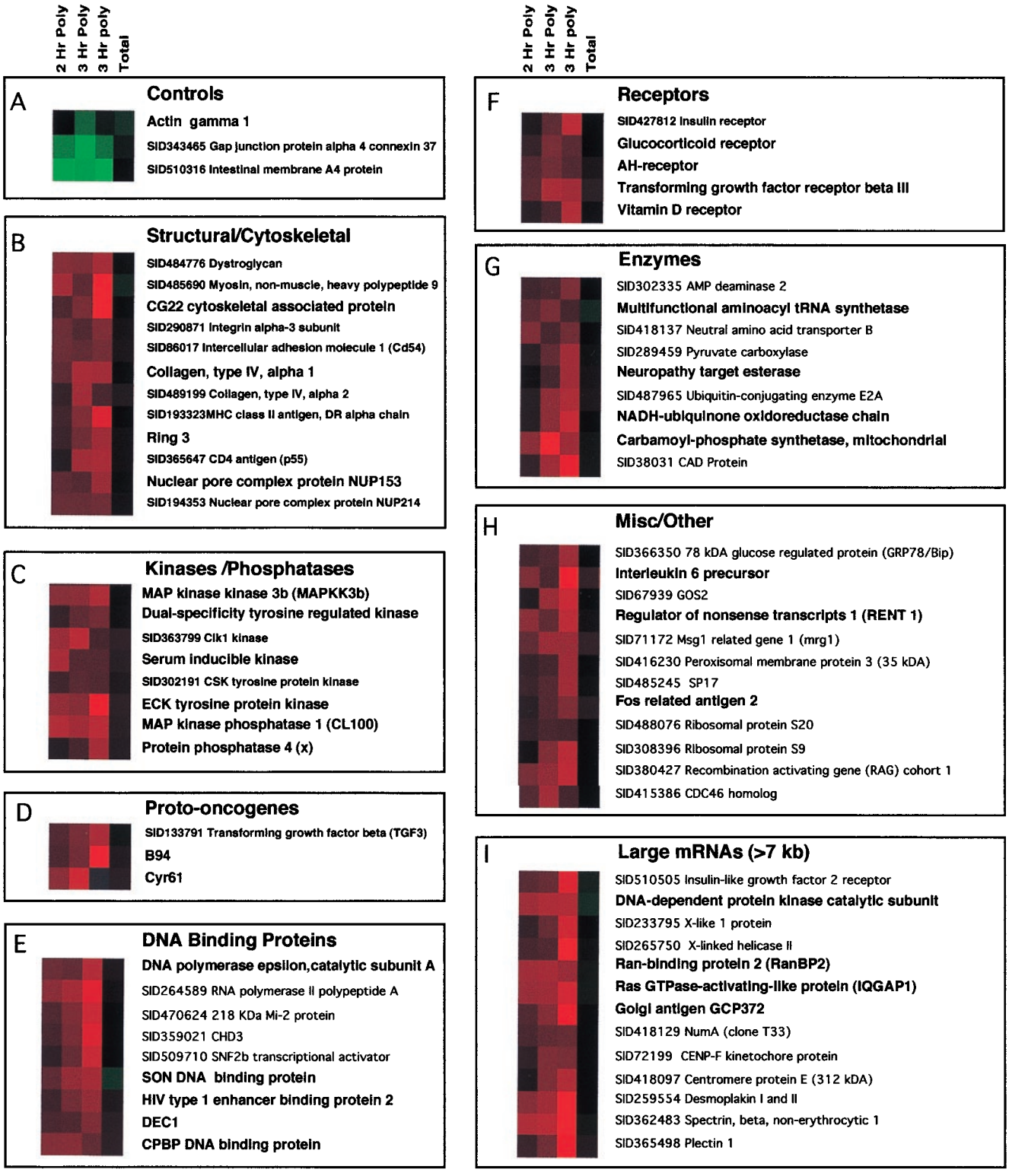

Fig. 2. Polysomal association of cellular mRNAs during poliovirus infection. Each colored square represents the ratio of total mRNA level in the cell (Total) or present on high molecular weight polysomes (Hr Poly) at different times after infection, relative to the same mRNA level in mock-infected cells (28). Black squares denote no significant change in the amount of RNA in infected and mock-infected cells; red squares denote RNAs that are more abundant in polio-infected cells, and green squares denote RNAs that are more abundant in mock-infected cells. The intensity of the color is proportional to the $\log_2$ of the fold increase/decrease, with maximal intensity corresponding to an 8-fold increase/decrease. Variations in intensities result from different hybridization reactions and different batches of chips. Panel *A* shows three genes whose expression pattern typifies that found in ≥97% of the genes on the microarray. (*B–I*) Named genes whose RNA levels were enriched >2-fold in polysomes from infected cells (2 Hr and 3 Hr Poly, shown in two independent experiments) but displayed no significant changes in their steady state levels (Total) are grouped within the following categories: Structural and Cytoskeletal (*B*), Kinases and Phosphatases (*C*), Proto-oncogenes (*D*), DNA Binding Proteins (*E*), Receptors (*F*), Enzymes (*G*), Miscellaneous and Other (*H*), and mRNAs larger than 7 kilobases (kb) (*I*). The clones are identified by either a name or preceded by a Stanford identification number (SID); SID-marked genes have not yet been verified by sequencing. However, ongoing sequencing of the clones has shown that 80% of the clones can be successfully verified.

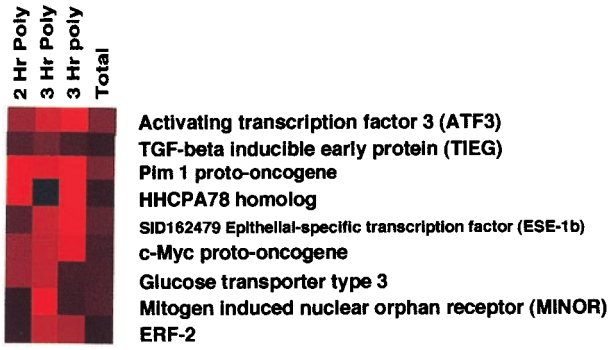

Fig. 3. Genes whose mRNAs levels increase in poliovirus-infected cells and that are also selectively enriched on polysomes in infected cells. Data are represented as detailed in the legend to Fig. 2.

nonpolysomal fractions 2–3 in gradients prepared from infected cells (Fig. 5). This redistribution reflected the poor translation of actin mRNA in infected cells (4). In contrast, c-myc mRNA redistributed from fraction 9 to fractions 7–9, reflecting the lack of change in translational efficiency of c-myc mRNA (4). Similarly, Pim-1, B94, Cl100, and MAPKK3b mRNAs sedimented preferentially with high molecular weight polysomes in both infected and uninfected cells (Fig. 5). Interestingly, Cyr61 mRNA was selectively mobilized into polysomes in infected cells (Fig. 5).

Pim-1 and Cyr61 mRNAs Contain IRES Elements. It is possible that many of the mRNAs that are polysome-associated in infected cells contain IRES elements. The 350-nt 5′ NCR of Pim-1 (10) and the 250-nt 5′NCR of Cyr61 (11) mRNAs are predicted to fold into a secondary structure with calculated free energies of −153 kcal/mol (14) and −83 kcal/mol, respectively (11). Thus, both mRNAs are predicted to be translated poorly by a 5′end-dependent scanning mechanism (15).

To test whether the 5′NCRs of Pim-1 and Cyr61 contain IRESs, plasmids expressing dicistronic RNAs, with various

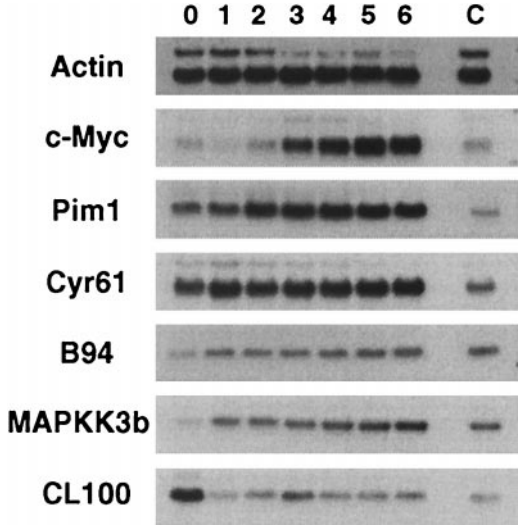

Fig. 4. Steady-state levels of cellular mRNAs during poliovirus infection. Total RNA was isolated at various times after infection of HeLa cells with poliovirus (0 to 6 hours) and was subjected to Northern blot analysis (5 μg per lane) by using radiolabeled DNA probes complementary to actin, c-myc, Pim-1, Cyr61, B94, MAPKK3b, and CL100. RNA isolated from mock-infected cells isolated at 3 hours is displayed in lane C. The fate of actin and c-myc mRNAs have previously been documented (4) and are included as controls.

sequence elements inserted between *Renilla* and firefly luciferase cistrons, were transfected into human HeLa cells. Fig. 6*A* shows that insertion of a landscape of RNA structures (ΔEMCV) between the two cistrons resulted in low synthesis of firefly luciferase compared with that of *Renilla* luciferase, indicating that readthrough of ribosomes from the first to the second cistron was infrequent. However, insertion of the 5′ NCR of either Pim-1 or Cyr61 upstream of the second cistron greatly stimulated the synthesis of firefly luciferase, indicating that these 5′NCRs provided functional IRES activities that were comparable to the activity usually observed with the encephalomyocarditis virus IRES (9). As shown by Northern blot analysis (Fig. 6*B*), the transcripts remained largely intact, arguing that the second cistron translation was unlikely to be initiated from spliced transcripts or from functionally monocistronic transcripts that may have been expressed from a promoter located in the 5′NCR-encoding DNA. Both Pim-1 and Cyr61 5′NCRs mediated translation of second cistrons in translation-competent lysates prepared from human HeLa cells (data not shown), which also argues against the presence of small amounts of functionally monocistronic mRNAs. Therefore, the 5′NCRs of Pim-1 and Cyr61 contain IRES elements that allow them to be translated at low eIF4F concentrations.

Discussion

By using cDNA microarrays to monitor the association of cellular mRNAs with polysomes, we discovered that as many as 3% of cellular mRNAs were associated with the translation apparatus in poliovirus-infected cells at a time when most of eIF4F was proteolyzed. These findings suggest that a significant class of cellular mRNAs can be translated at greatly reduced levels of intact eIF4F.

Polysomal Association of Cellular mRNAs with the Translation Apparatus in Infected Cells. Several possible models could explain the association of mRNAs with polysomes in infected cells. For example, the ribosomal transit time could be slowed for some mRNAs, or a block in elongation could retain selected mRNAs on polysomes. More likely, many of the mRNAs were associated with polysomes in infected cells because of a reduced requirement for eIF4F in translational initiation. The latter hypothesis is substantiated by the findings that both mRNAs with unstructured 5′NCRs (6, 16) and mRNAs with structured IRES elements in their 5′NCRs are translated in poliovirus-infected cells (4). Several known IRES-containing mRNAs, such as BiP and c-myc (4), were represented on polysomes from infected cells. A notable exception was the IRES-containing vascular endothelial growth factor VEGF mRNA, which was not associated with polysomes under these conditions. Overall, these findings indicated that several mRNAs with known and novel IRES elements, such as those present in Cyr61 and Pim-1, could be detected on polysomes from infected cells by using the cDNA display. Whether these IRES elements need small amounts of eIF4GII-containing eIF4F complexes for internal ribosome recruitment remains an unprecedented yet interesting possibility.

MAPKK3b and MAP Kinase Phosphatase 1 (MKP-1). Several of the polysome-associated mRNAs encoded gene products of the highly conserved MAP kinase pathways and products that have roles in inflammation, angiogenesis, and response of cells to extracellular signals such as serum deprivation. There are at least three distinct MAP kinase pathways in mammalian cells, including the extracellular signal-regulated kinase cascades that are regulated by Ras and the stress-activated MAP kinase pathways defined by JNK and p38 (17). The extracellular signal-regulated kinase pathways mainly respond to mitogens and growth factors whereas the stress-activated MAP kinase

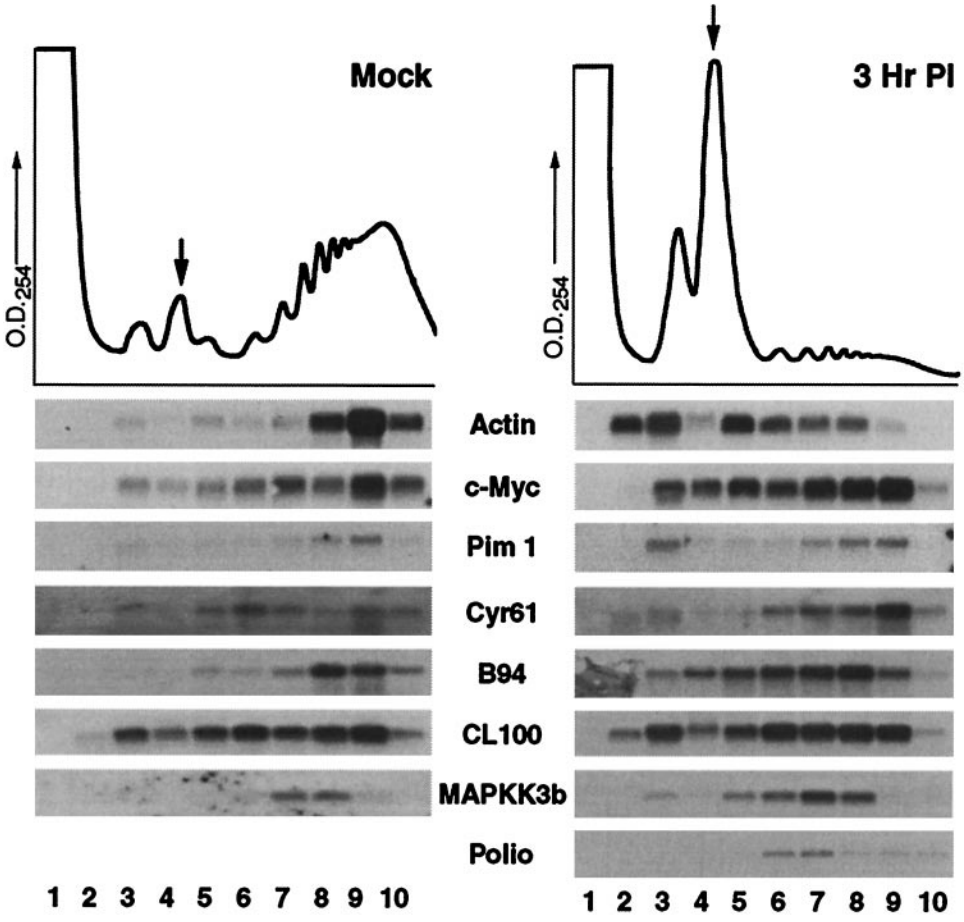

Fig. 5. Polysomal distribution of specific cellular mRNAs. Polysomal distributions, visualized after sucrose gradient sedimentation, of cellular mRNAs from mock- (Mock) and poliovirus-infected cells at 3 hours after infection (3 Hr PI) are shown. Absorbance profiles at 254 nm of the collected sucrose gradients are shown at the top of each panel. Ten fractions were collected from each gradient (fraction 1 is the top of the gradient), and equal volumes of each fraction were separated on a formaldehyde-agarose gel and subjected to Northern blot analysis using specific radiolabeled DNA probes. The sedimentation profiles of c-Myc, actin, and polioviral (polio) mRNAs have been shown previously (4) and are included as controls in this study.

pathways are activated by environmental changes and by inflammatory cytokines such as tumor necrosis factor α and IL-1. None of several extracellular signal-regulated kinase kinase mRNAs (i.e., MEK2, MEK5, extracellular signal-regulated kinase 3) (17) were found to be associated with polysomes in infected cells (not shown). However, this study has identified an eIF4F- independent polysomal association of MAPKK3b (18) and MKP-1 (19) mRNAs whose gene products are some of the known regulators of the p38 MAP kinase. If the polysomal association of MAPKK3b mRNA in infected cells results in the synthesis of MAPKK3b protein, activation of its p38 substrate could lead to activation of downstream targets required for the production of inflammatory cytokines, such as IL-12 (20). Among the substrates for MAPKK3b are also the Mnk1 and Mnk2 kinases (21, 22). Recently, Mnk1 was shown to be associated with eIF4G in the eIF4F complex, and the cap binding protein eIF4E is a Mnk1 substrate (23). Based on studies on the three-dimensional structure of eIF4E, it was speculated that phosphorylation of eIF4E could stabilize the interaction of eIF4F with the cap structure (23). Thus, one could envisage that translation of the p38 activator MAPKK3b, at times when functional eIF4F is limiting, could lead to activation of Mnk1. Mnk1 phosphorylation of eIF4E could result in the production of functional eIF4F complexes.

MAP kinase phosphatase 1, MKP-1 or CL100, is an immediate early gene whose transcription is induced within 1 hour after exposure of cells to growth factors or to oxidative stress (19). Substrates for MKP-1 include the kinases of the stress-activated MAP kinase pathways, especially the p38 kinase (24). This study shows MKP-1 mRNA remained associated with polysomes in poliovirus-infected cells. Recently, we could demonstrate that the 5′NCR of MKP-1 provides functional IRES activity in dicistronic constructs (M.S.C., unpublished work). Thus, mRNAs encoding both a kinase (MAPKK3b) and a phosphatase (MKP-1) for p38 can be translated at reduced eIF4F concentrations.

Cyr61. Cyr61 encodes a serum-induced secreted protein that induces the movement of vascular endothelial cells in culture and induces tumor growth and angiogenesis *in vivo* (25). Cyr61 mRNA was associated with polysomes in poliovirus-infected cells and, like polioviral mRNA, contains a functional IRES in its 5′NCR. The observed IRES activity was remarkable in that the 5′ terminal half of the 5′NCR of Cyr61 was not included in the IRES assay.

Pim-1. The Pim-1 gene encodes a serine-threonine protein kinase that can cooperate with c-myc in cellular transformation, predominantly in hematopoietic cells (10, 26). The 5′NCR of Pim-1 is GC-rich and predicted to be highly structured. This leader element greatly inhibits mRNA translation in the rabbit reticu-

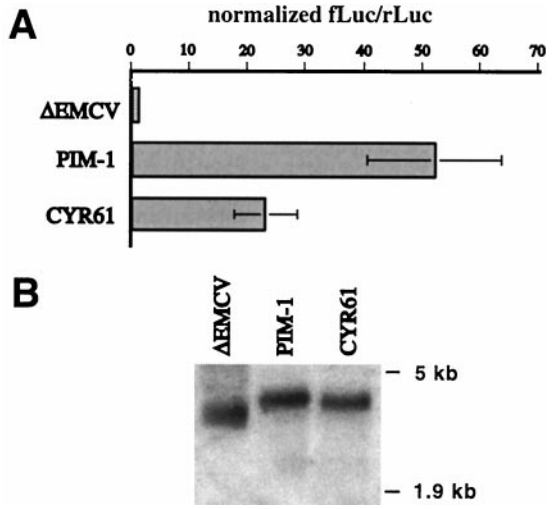

Fig. 6. Test for IRES activity in the 5′ NCRs of Pim-1 and Cyr61 mRNAs. (*A*) Expression of dicistronic mRNAs containing ΔEMCV sequences or the 5′NCRs of Cyr61 or Pim-1 in the intercistronic spacer regions. Plasmid DNA (5 μg) and 25 μg of lipofectin reagent (GIBCO/BRL) were mixed and transfected into HeLa cells. The results are displayed as the ratio of second cistron fLuc to first cistron rLuc activity. The ratios of luciferase activities obtained from transfection experiments with the control plasmid pRΔDEF was set to 1. Results from three independent transfections were averaged and are shown. (*B*) Integrity of dicistronic mRNAs in transfected cells. Poly(A+) mRNA was isolated from cells transfected with dicistronic plasmids and was analyzed by Northern blot hybridization using radiolabeled DNA probes complementary to firefly luciferase sequences. The migrations of 28S and 18S rRNA are indicated.

locyte lysate and in cultured cells (14), implying that the mRNA is translated by a 5′end-dependent scanning mechanism in these experimental systems. Overexpression of eIF4E in cultured cells greatly relieved the inhibitory effect of the Pim-1 5′NCR on translation (14). Our study shows that the Pim-1 5′NCR has strong IRES activity in a dicistronic construct and within natural Pim-1 mRNA. The stimulation of translation of an IRES-containing mRNA by eIF4E is puzzling. It is tempting to speculate that modification of translation initiation factors may play a role in both 5′ end dependent and internal initiation mechanisms in Pim-1 mRNA. For example, the Mnk1 kinase could enhance the assembly of eIF4F both at the 5′ end of a mRNA via phosphorylation of eIF4E (23) and at an IRES via phosphorylation of eIF4G. Indeed, it has been pointed out that eIF4G is also a substrate for Mnk1 (23).

The genes we found to associate with polysomes under conditions of limiting eIF4F were highly enriched for genes whose transcriptions have previously been shown to be induced by serum and whose products have roles in inflammation and angiogenesis (8). This common functional and regulatory theme suggests that these genes may be subject to selective translational induction.

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